

Chronic cat allergen exposure induces a T_H2 cell-dependent IgG₄ response related to low sensitization

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Background: In human subjects, allergen tolerance has been observed after high-dose allergen exposure or after completed allergen immunotherapy, which is related to the accumulation of anti-inflammatory IgG₄. However, the specific T-cell response that leads to IgG₄ induction during chronic allergen exposure remains poorly understood.

Objective: We sought to evaluate the relationship between cat allergen-specific T-cell frequency, cat allergen-specific IgE and IgG₄ titers, and clinical status in adults with cat allergy with and without cat ownership and the cellular mechanism by which IgG₄ is produced.

Methods: Fel d 1–, Fel d 4–, Fel d 7–, and Fel d 8–specific T-cell responses were characterized by CD154 expression after antigen stimulation.

Results: In allergic subjects without cat ownership, the frequency of cat allergen (Fel d 1 and Fel d 4)–specific T_H2 (sT_H2) cells correlates with higher IgE levels and is linked to asthma. Paradoxically, we observed that subjects with cat allergy and chronic cat exposure maintain a high frequency of sT_H2 cells, which correlates with higher IgG₄ levels and low sensitization. B cells from allergic, but not nonallergic subjects, are able to produce IgG₄ after cognate interactions with sT_H2 clones and Fel d 1 peptide or the Fel d 1 recombinant protein.

Conclusion: These experiments suggest that (1) allergen-experienced B cells with the capacity to produce IgG₄ are present in allergic subjects and (2) cat allergen exposure induces an IgG₄ response in a T_H2 cell–dependent manner. Thus IgG₄ accumulation could be mediated by chronic activation of the

T_H2 response, which in turn drives desensitization. (*J Allergy Clin Immunol* 2015;■■■:■■■–■■■.)

Key words: Cat allergy, Fel d 1, Fel d 4, T_H2 cells, allergen tolerance, asthma, class II tetramer, CD154, IgG₄, allergen exposure

An allergic reaction is characterized by a type 2 immune response in which CD4⁺ T_H2 cells produce IL-4, IL-5, IL-13, IL-9, and IL-10; and allergen-specific IgE which binds to high-affinity IgE receptors (FcεRI) on basophils and mast cells to enhance histamine secretion and the allergic response.¹ Although the pathologic T_H2 response against allergen is well characterized, the nature of tolerant responses is less well understood. Naturally occurring allergen tolerance has been described in subjects with cat allergy, with a correlation between cat allergen exposure and reduced sensitivity observed in children owning a cat.²

Reduced sensitivity in subjects with cat ownership is associated with the accumulation of specific IgG₄, which has the capacity to attenuate IgE-mediated allergic symptoms.^{3–6} IgG₄ accumulation is also observed in tolerant beekeepers during the bee sting season, confirming an important role for this antibody in allergen tolerance.⁷ Allergen-specific T cells that produce IL-10 are observed in both cat owners and beekeepers and likely play a role in specific IgG₄ induction.^{8,9} Furthermore, induction of IL-10 and specific IgG₄ is correlated with improved clinical outcome during allergen-specific immunotherapy.^{10–12} Although the importance of these factors in the allergen tolerance mechanism is therefore evident, the role of T-cell subsets and other factors supporting specific IgG₄ production remains unclear.

Although IgE detection is routinely used to discriminate allergen sensitivity, characterization of allergen-specific CD4⁺ T cells is more difficult. For subjects with cat allergy, the situation is further complicated by the presence of multiple allergens. Fel d 1 is recognized as the major cat allergen, and 80% to 95% of subjects with cat allergy have IgE that recognizes Fel d 1¹³; however, IgE to Fel d 4, Fel d 7, and Fel d 8 has been reported to be present in 60%, 40%, and 20% of subjects with cat allergy, respectively.^{14,15} All of these allergens can be major CD4⁺ T-cell targets in subjects with cat allergy.¹⁶

Peptide–MHC class II tetramer staining is one of the most sensitive methods to characterize antigen-specific CD4⁺ T cells during inflammatory diseases and allergy.^{17–19} Detection of CD154 expression after antigen stimulation has also been used to track allergen-specific CD4⁺ T cells.^{19–21} In the present study we used these T-cell tracking methods to characterize Fel d 1–, Fel d 4–, Fel d 7–, and Fel d 8–specific T-cell responses in adults with cat allergy with and without cat ownership. The relationship between cat allergen-specific T-cell frequency, cat dander-specific IgE and specific IgG₄ titers, and clinical status

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Abbreviations used

APC: Allophycocyanin
 CRTH2: Chemoattractant receptor–homologous molecule expressed on T_H2 cells
 PE: Phycoerythrin
 sT_H2: Specific memory CD4⁺ T_H2
 T_{FH}: T follicular helper

was examined, and the cellular mechanism that led to high IgG₄ production in subjects with cat allergy was investigated. Here we demonstrate that chronic cat allergen exposure supports a T_H2 cell–dependent IgG₄ response.

METHODS**Subjects**

Thirty-eight subjects were recruited from the Virginia Mason Medical Center Allergy Clinic and Benaroya Research Institute. All subjects were HLA typed (see the [Methods](#) section in this article's Online repository at www.jacionline.org). Subjects with cat allergy (n = 24) had a positive skin test response, serum IgE response, or both to cat dander of greater than 0.35 kU_A/mL, as determined by using the ImmunoCAP test (Phadia AB, Uppsala, Sweden), and had known allergic symptoms. Nonallergic subjects (n = 14) had negative IgE ImmunoCAP scores for cat allergens and other allergens (dust mite, Bermuda grass, timothy grass, alder, and *Alternaria* species). All subjects were recruited after obtaining informed consent and institutional review board approval. All assays were performed in a blinded manner with regard to cat ownership status and allergic scores. Asthma and rhinitis scores were based on symptoms and medications, as described in the [Methods](#) section in this article's Online repository.

Ex vivo analysis of cat allergen–specific CD4⁺ T cells

For the CD154 expression assay, 30 × 10⁶ PBMCs (at 6 × 10⁶ cells/mL) were stimulated for 3 hours at 37°C with 5 μg/mL synthesized peptide (20 amino acids in length with a 12-amino-acid overlap) pools (Mimotopes, Notting Hill, Australia) spanning the entire Fel d 1 (p1-p18), Fel d 4 (p1-p20), Fel d 7 (p1-p18), and Fel d 8 (p1-p27) sequences or with dimethyl sulfoxide (control) in the presence of 1 μg/mL anti-CD40 (HB14; Miltenyi Biotec, Bergisch Gladbach, Germany) in 10% human serum RPMI medium. After stimulation, PBMCs were labeled with phycoerythrin (PE)–conjugated CD154 and CD154⁺ cells and were enriched with anti-PE magnetic beads (Miltenyi Biotec). A one-tenth fraction of unenriched cells was saved for analysis for frequency determination (see the [Methods](#) section in this article's Online repository). After enrichment, cells were stained with appropriate antibodies (see the [Methods](#) section in this article's Online repository). Staining with HLA-DRB1*0301/Fel d 1 chain 2₁₇₋₃₆ and HLA-DRB5*0101/Fel d 1 chain 1₁₇₋₃₆ tetramers was carried out, as previously described.¹⁸

Intracellular cytokine staining

For *ex vivo* intracellular cytokine staining combined with the CD154 expression assay, monensin was added during stimulation (GolgiStop; BD Biosciences, San Jose, Calif), according to the manufacturer's instructions. *In vitro* intracellular cytokine staining combined with MHC class II tetramer staining was performed, as previously described.¹⁹ Cells were stained with appropriate antibodies (see the [Methods](#) section in this article's Online repository).

Specific and total IgG₄ analysis

The determination of Fel d 1– and Fel d 4–specific IgG₄ antibody levels in adult serum was performed by using ELISA, as described previously.²²

Briefly, either 1 μg/mL recombinant Fel d 1 or Fel d 4 (prepared as previously described^{15,16}) or 1% BSA (as a control) was coated on wells. After washing, one-tenth–diluted sera from patients were added to wells coated with Fel d 1 or Fel d 4 or 1% BSA. The detection methods are as described in the [Methods](#) section in this article's Online repository. Total IgG₄ levels were quantified in culture supernatants by using Human IgG4 Ready-SET-Go (eBioscience, San Diego, Calif), according to the manufacturer's instructions.

Fel d 1–specific T_H2 clone generation

After staining with DRB5*0101/Fel d 1 chain 1₁₇₋₃₆ tetramers or DRB1*0301/Fel d 1 chain 2₁₇₋₃₆, single memory (CD45RA⁺) tetramer-positive cells were sorted *ex vivo* in a 96-well plate on an FACSria II flow cytometer (BD Biosciences) by using FACSDiva software (BD Biosciences). Single cells were expended, as described in the [Methods](#) section in this article's Online repository. Positives clones were selected based on their capacity to bind Fel d 1 tetramer compared with unloaded tetramer (empty tetramer).

B-cell and PBMC cultures

B cells were isolated from PBMCs by using an indirect magnetic labeling system (B cell Isolation Kit II, Miltenyi Biotec). These cells were more than 90% CD19⁺. B cells (2 × 10⁵) were cultured with 10⁵ T_H2 clones for 14 days in the presence or absence of the appropriate Fel d 1 peptide in 10% FBS RPMI medium. For culture with the recombinant Fel d 1 protein (40 μg/mL), 2 × 10⁵ purified B cells with or without 10⁵ T_H2 clones were cultured in the presence or absence of recombinant Fel d 1 protein (40 μg/mL) with or without 10⁵ PBMCs depleted on B cells in 10% FBS RPMI medium. Supernatants were collected after 14 days. Cells were stained after 14 days of culture with appropriate antibodies (see the [Methods](#) section in this article's Online Repository).

Statistical analysis

Statistical analysis was performed with the tests indicated in the figure legends by using Prism 5.0 software (GraphPad Software, La Jolla, Calif).

RESULTS**Fel d 1– and Fel d 4–specific T_H2 cells are predominant during cat allergy**

Specific T-cell responses to Fel d 1, Fel d 4, Fel d 7, and Fel d 8 were evaluated by using the CD154 upregulation assay, as described in the [Methods](#) section ([Fig 1, A](#), and see [Fig E1](#) in this article's Online Repository at www.jacionline.org). The CD154 assay accurately measures antigen-specific T cells because a direct comparison of the Fel d 1 CD154 assay and Fel d 1 tetramer assay yielded similar results (see [Fig E2](#) in this article's Online Repository at www.jacionline.org).^{18,19} Stimulation of PBMCs from subjects with cat allergy with cat allergen peptides led to detection of high frequencies of memory, but not naive, Fel d 1– and Fel d 4–specific CD154⁺CD4⁺ T cells compared with those in the nonstimulated control and nonallergic subjects ([Fig 1, A and B](#), and see [Figs E3 and E4](#) in this article's Online Repository at www.jacionline.org). A high T-cell response to Fel d 4 could be found irrespective of a high Fel d 1–specific T-cell response (see [Fig E4, A and B](#)), and there was no correlation between Fel d 1– and Fel d 4–specific T-cell responses (see [Fig E4, C](#)). Thus the combination of Fel d 1– and Fel d 4–specific T-cell responses covers the majority of the cat allergen–specific T-cell response in allergic subjects ([Fig 1, C](#)).

The high-frequency Fel d 1– and Fel d 4–specific memory T cells in allergic subjects were found to be chemoattractant

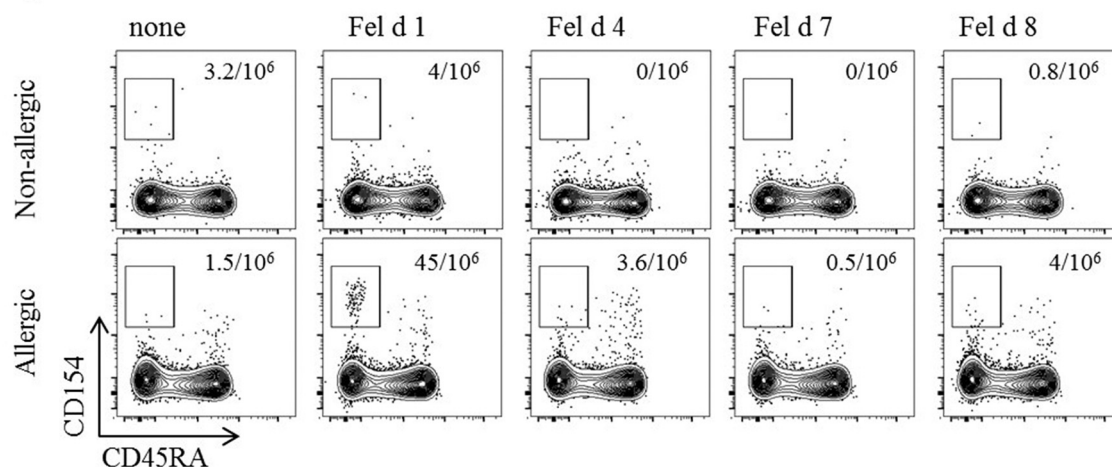
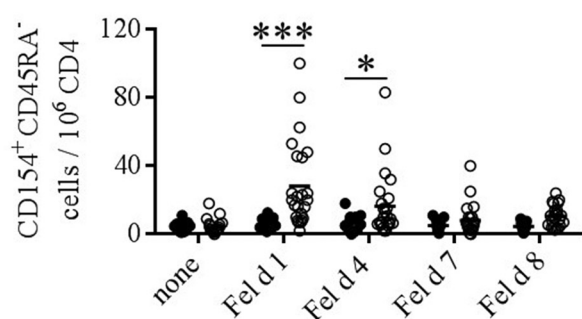
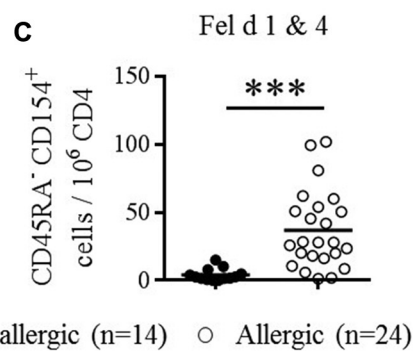
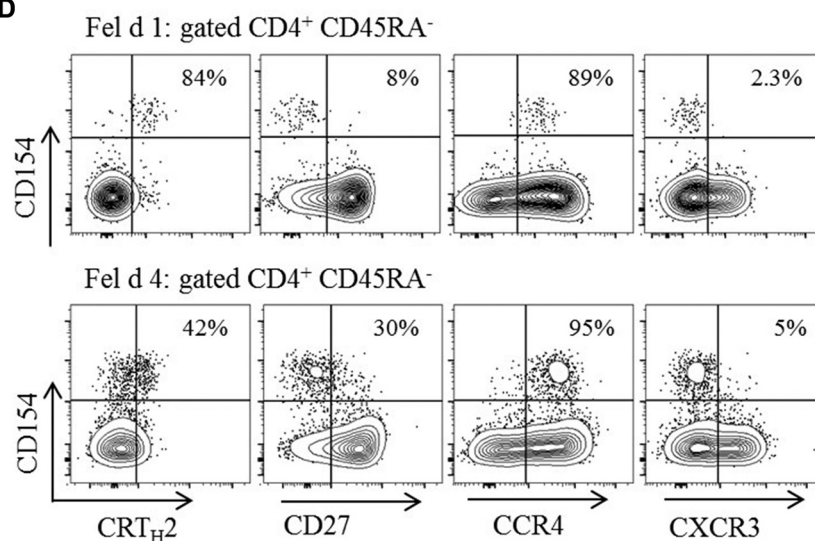
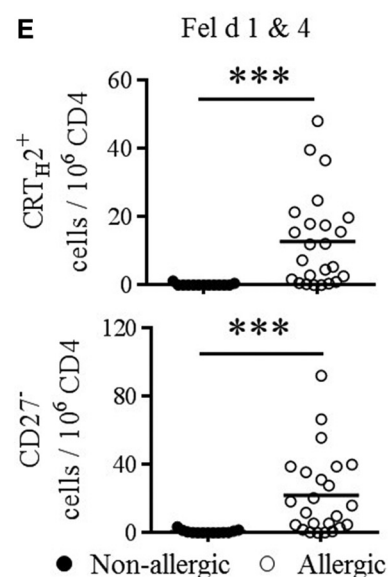
A gated CD4⁺:**B****C****D****E**

FIG 1. Fel d 1- and Fel d 4-specific memory T_H2 cells are predominant during cat allergy. **A**, CD154 expression after stimulation with cat allergen peptides or dimethyl sulfoxide (*none*). Numbers indicate the frequency of memory CD45RA⁻ CD154⁺ T cells per 10⁶ CD4⁺ T cells. **B** and **C**, Frequency of Fel d 1, Fel d 4, Fel d 7, and Fel d 8 (Fig 1, **B**) and combined Fel d 1 and Fel d 4 (Fig 1, **C**) memory CD154⁺ T cells after cat allergen peptide stimulation. **D**, CRTH2, CD27, CCR4, CXCR3, and CD154 expression on gated memory Fel d 1- or Fel d 4-specific T cells from 2 different allergic adults. The percentage of each marker on CD154⁺ T cells is as indicated. **E**, Frequency comparison of combined Fel d 1- and Fel d 4-specific memory CD154⁺ CRTH2⁺ (top) or CD27⁻ (bottom) T cells between allergic and nonallergic subjects. Data were compared by using the Mann-Whitney test. **P* ≤ .05 and ****P* ≤ .001.

TABLE I. Subject information

Samples	HLA-DR		Asthma	Rhinitis score	Cat owner	Age of first ownership (y)	Current age (y)	SPT	IgE (kU/L)
Allergy#1	404	1302	Yes	Mild	No		36	3	6.35
Allergy#2	301	1501	Yes	Moderate	No		32	4	23.6
Allergy#3	701	1501	Yes	Mild	No		32	4	3.91
Allergy#4	901	1501	Yes	Severe	No		42	4	33.8
Allergy#5	401	701	Yes	Moderate	No		37	3	72.3
Allergy#6	301	401	Yes	NA	No		28	4	7.59
Allergy#7	1401	404	Yes	Moderate	No		66	NA	25
Allergy#8	1001	405	Yes	Moderate	No		34	4	20.8
Allergy#9	1501	1301	Yes	Moderate	No		35	3	4.94
Allergy#10	1101	1501	No	Moderate	No		54	4	0.49
Allergy#11	301	401	No	Moderate	No		61	4	5.65
Allergy#12	1001	1404	No	Moderate	No		39	4	1.28
Allergy#13	1101	1502	No	Moderate	No		30	4	4.42
Allergy#14	101	1501	No	Moderate	No		46	4	0.96
Allergy#15	1101	1501	No	Moderate	No		57	4	3.88
Allergy#16	401	1301	Yes	Mild	Yes	17	48	4	3.59
Allergy#17	301	401	Yes	Mild	Yes	8	46	3	12.5
Allergy#18	301	801	Yes	Mild	Yes	6	50	2	6.42
Allergy#19	701	1301	Yes	Moderate	Yes	0	36	2	58.3
Allergy#20	701	1201	Yes	Moderate	Yes	0	67	4	7.1
Allergy#21	301	407	No	Mild	Yes	0	34	2	9.03
Allergy#22	701	1101	No	Mild	Yes	3	39	3	11.4
Allergy#23	1501	701	No	Mild	Yes	5	36	2	21.3
Allergy#24	401	1101	No	Mild	Yes	3	32	3	8.41
Control#1	701	1302	—		No		42	NA	0
Control#2	701	801	—		No		32	NA	0
Control#3	1104	1104	—		No		30	NA	0
Control#4	407	102	—		No		30	NA	0
Control#5	401	301	—		No		30	NA	0
Control#6	701	1302	—		No		34	NA	0
Control#7	106	1501	—		No		30	NA	0
Control#8	1501	1501	—		No		32	NA	0
Control#9	301	1104	—		Yes	29	33	NA	0
Control#10	301	401	—		Yes	26	37	NA	0
Control#11	404	1601	—		Yes	0	31	NA	0
Control#12	404	1301	—		Yes	7.5	66	NA	0
Control#13	401	1501	—		Yes	20	28	NA	0
Control#14	101	103	—		Yes	0	34	NA	0

NA, Not available; SPT, skin prick test (0-4+).

receptor-homologous molecule expressed on T_H2 cells (CRTH2)⁺ and CD27⁺ T cells and correspond to an effector T_H2 phenotype (Fig 1, D and E).^{17,23} Moreover, accumulation of allergen-specific memory CD4⁺ T cells is directly linked to the effector T_H2 phenotype (see Fig E5 in this article's Online Repository at www.jacionline.org). A relatively high frequency of Fel d 7-specific memory CD4⁺ T_H2 cells (sT_H2 cells) was also observed in 2 of 24 subjects examined (see Figs E4 and E5), but the overall response of the group was not significantly different.

Cat exposure correlates with low sensitization and a relatively high frequency of allergen-specific CD4⁺ T_H2 cells in allergic subjects

Nine allergic adults in this study were cat owners and therefore constantly exposed to cat allergens (Table I). We found that allergic adults with cat ownership and allergic adults without a

TABLE II. Influence of cat exposure on sensitization, IgE levels, and clinical symptoms

	Nonallergic	Allergic		P value
		Cat owner	Without a cat	
No. of samples	14	9	15	
Skin prick test (0-4+)	ND	2.8 ± 0.3	3.8 ± 0.1	.0009*
IgE (kU/L)	0	15.3 ± 5.6	14.3 ± 4.9	.8974*
No asthma	—	44.5% (n = 4)	40% (n = 6)	
Asthma	—	55.5% (n = 5)	60% (n = 9)	
Odds (yes/no)		1.25	1.5	1†
Mild rhinitis	0	78% (n = 7)	13% (n = 2)	
Moderate rhinitis	0	22% (n = 2)	73% (n = 11)	
Severe rhinitis	0	0	6% (n = 1)	
Odds (moderate or severe/mild)		0.3	6	.0066†

P values with statistical significance are shown in boldface.

*Student unpaired t test.

†Fisher exact test.

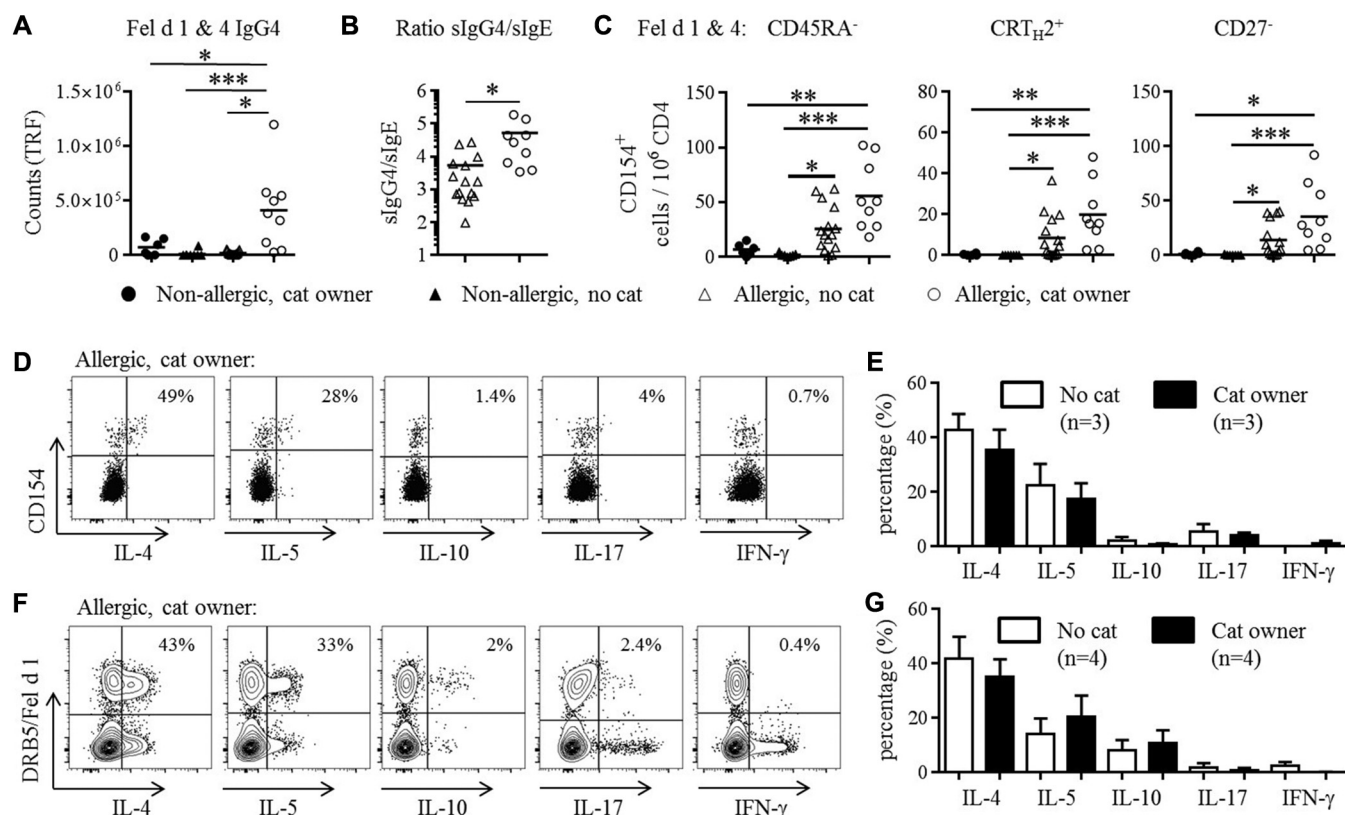


FIG 2. Cat allergen exposure induces specific IgG₄ and allergen-specific CD4⁺ T_H2 cell responses in allergic adults. **A**, Combined Fel d 1- and Fel d 4-specific IgG₄ levels in the serum of nonallergic and allergic adults. **B**, Specific IgG₄/specific IgE ratio (log-transformed) in subjects with and without cat ownership. **C**, Combined frequency of Fel d 1- and Fel d 4-specific memory CD45RA⁻, CRT_H2⁺, or CD27⁻ CD154⁺ T cells. **D**, CD154 and cytokine expression on gated CD4⁺ T cells after Fel d 1 peptide stimulation. **E**, Comparison of cytokine expression of CD154⁺ T cells in allergic adults. **F**, Intracellular cytokine staining of DRB5*0101 (DRB5)-restricted Fel d 1 chain 17-36-specific CD4⁺ T cells in a DRB5 cat-owning allergic adult by using tetramer staining. Percentages of IL-4⁺, IL-5⁺, IL-17⁺, IFN-γ⁺, and IL-10⁺ Fel d 1-specific CD4⁺ T cells are indicated. **G**, Cytokine expression of tetramer-positive Fel d 1-specific CD4⁺ T cells in allergic adults. Data were compared by using the Student unpaired *t* test (Fig 2, B), the Kruskal-Wallis test with the Dunn multiple comparisons test (Fig 2, A and C), or the Mann-Whitney test (Fig 2, E and G). **P* ≤ .05, ***P* ≤ .01, and ****P* ≤ .001.

cat at home had similar cat dander-specific IgE levels (Table II); however, cat owners had significantly lower skin prick test scores (Table II). Cat-owning allergic adults also had significantly lower rhinitis symptom severity (78% with mild symptoms and 22% with moderate symptoms) than allergic adults without a cat (13% with mild symptoms, 73% with moderate symptoms, and 6% with severe symptoms). However, regarding asthma prevalence, no significant difference were observed between these 2 allergic groups.

In this study cat-owning allergic adults had significantly higher levels of serum Fel d 1- and Fel d 4-specific IgG₄ than nonallergic adults with or without a cat or allergic adults without a cat (Fig 2, A; see Fig E6 in this article's Online Repository at www.jacionline.org). Moreover, the specific IgG₄/specific IgE ratio was significantly higher in allergic adults who own cat versus that seen in allergic adults who did not own a cat (Fig 2, B). Allergic adults with cat ownership also had a higher frequency of Fel d 1 and Fel d 4 sT_H2 cells than nonallergic adults with or without a cat (Fig 2, C; Fig E6). Allergic cat owners also had a higher frequency of Fel d 1 and Fel d 4 T cells than allergic

subjects without a cat, although the difference was not statistically significant. However, the IgG₄ accumulation observed in cat-owning allergic adults was not associated with a shift of T_H2 cells to CXCR5⁺ T follicular helper (T_{FH}) cells, which are known to support IgG production (see Fig E7 in this article's Online Repository at www.jacionline.org).^{24,25}

The observed T_H2 phenotype of cat allergen-specific T cells in allergic adults with cat ownership (Fig 2, C) is supported by *ex vivo* and *in vitro* cytokine secretion, in which Fel d 1-specific memory CD4⁺ T cells primarily secreted IL-4 and IL-5. No difference in the cytokine profile of sT_H2 cells was observed between allergic groups (Fig 2, D-G).

sT_H2 cell response correlates with the allergen immunoglobulin response

In non-cat-owning allergic adults, sT_H2 cell frequency correlated with cat dander-specific IgE levels but not with specific IgG₄ levels (Fig 3, A and B). In contrast, in cat-owning allergic adults the sT_H2 frequency correlated with specific IgG₄

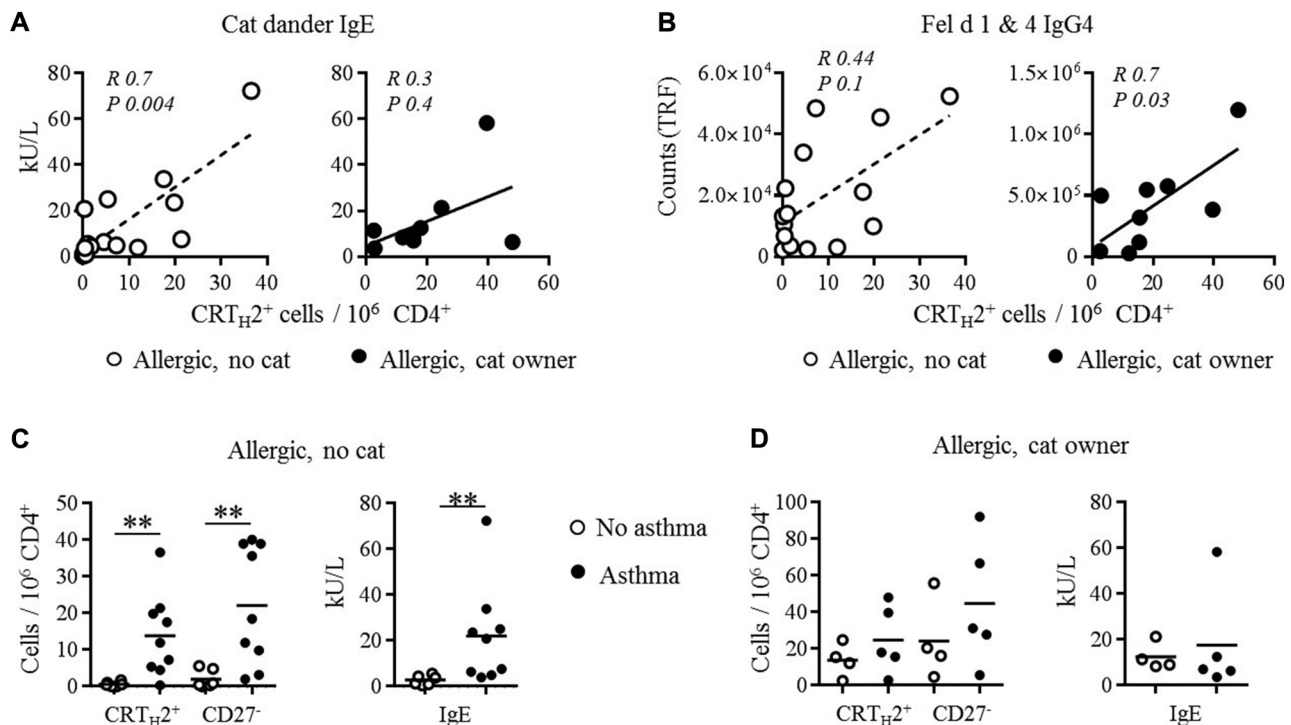


FIG 3. Correlation between the T_{H2} cell response and specific IgE or specific IgG₄ level depends on allergen exposure. **A** and **B**, Correlation between the combined frequency of Fel d 1– and Fel d 4–specific memory CD154⁺CRTH2⁺CD45RA[−]CD4⁺ T cells and the total cat dander-specific IgE level (Fig 3, **A**) or the combined Fel d 1– and Fel d 4–specific IgG₄ level (Fig 3, **B**) in allergic adults with or without a cat. TRF, Time-resolved fluorometry. **C** and **D**, Comparison of the combined frequency of Fel d 1– and Fel d 4–specific memory CD154⁺CRTH2⁺ or CD27[−] cells and specific IgE levels between asthmatic and nonasthmatic allergic adults without (Fig 3, **C**) or with (Fig 3, **D**) a cat. Spearman R and P values were determined to analyze the correlation. Data were compared by using the Mann-Whitney test. ** $P \leq .01$.

but not specific IgE levels (Fig 3, **A** and **B**). In our non-cat-owning allergic cohort, we observed a link between asthma prevalence, sT_{H2} cell frequency, and specific IgE levels (Fig 3, **C**). In contrast, there was no link between asthma and sT_{H2} cell frequency or IgE levels in cat-owning allergic subjects (Fig 3, **D**). These results demonstrate that cat allergen exposure modulates the relationship between the T_{H2} response, specific IgE levels, and asthma prevalence. Moreover, these results suggest a link between the T_{H2} response and IgG₄ accumulation for subjects with cat allergy and cat ownership.

Fel d 1 sT_{H2} cells support IgG₄ production by B cells from allergic subjects

We tested the hypothesis that T_{H2} cells can support IgG₄ production. For evaluation of the capacity of T_{H2} cells to induce IgG₄ in the absence of chronic allergen exposure, single-cell sorting and cloning of Fel d 1 tetramer–positive memory cells were performed to generate Fel d 1 sT_{H2} clones from allergic subjects without cat ownership (see Fig E8 in this article's Online Repository at www.jacionline.org). Fel d 1 sT_{H2} clones were cultured with isolated B cells from allergic (with or without cat ownership) and nonallergic subjects without cat ownership (with matching HLA to the T_{H2} clones used in the experiment) in the presence of the appropriate Fel d 1 peptide to induce a cognate T-cell/B-cell response (Fig 4). After 14 days of T- and B-cell coculture, total IgG₄ levels were detected in cultures with sT_{H2} clones and B cells from allergic

adults (Fig 4, **A** and **B**) but not in those with B cells from nonallergic adults (Fig 4, **C**). CD38 expression was upregulated on the surfaces of sT_{H2} clones and B cells in the presence of specific peptide in cultures with B cells from both allergic and nonallergic subjects (Fig 4, **D–F**; see Fig E9 in this article's Online Repository at www.jacionline.org), demonstrating the activation of these cells during the cognate immune response.

Because chronic allergen exposure can influence T-cell function, we also tested the ability of sT_{H2} clones from allergic subjects with cat ownership to induce IgG₄. In accordance with previous observations (Fig 2, **D–G**), sT_{H2} clones from allergic subjects with and without cat ownership had similar T_{H2} cytokine profiles (see Fig E10 in this article's Online Repository at www.jacionline.org). Moreover, sT_{H2} clones from allergic subjects with cat ownership had similar capacity to induce IgG₄ production by B cells compared with sT_{H2} clones from allergic subjects without cat ownership (see Fig E11 in this article's Online Repository at www.jacionline.org).

These experiments demonstrated the capacity of T_{H2} cells to support IgG₄ production by B cells of allergic subjects but not those of nonallergic subjects.

The Fel d 1 recombinant protein enhances IgG₄ production in a T_{H2} cell–dependent manner

Asthma status and chronic allergen exposure are both linked to a T_{H2} response (Figs 2 and 3). However, the immunoglobulin

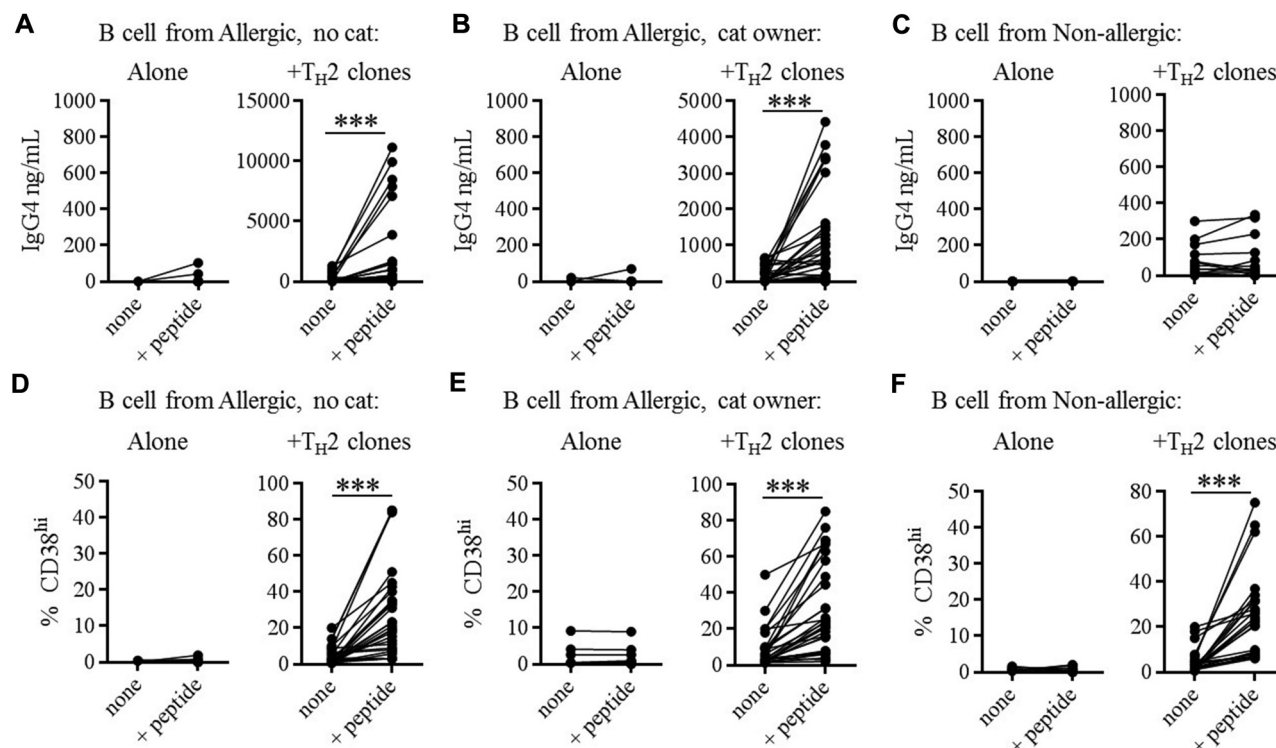


FIG 4. Fel d 1 sT_H2 cells support IgG₄ production by allergen-experienced B cells. **A-C**, Total IgG₄ levels in the culture supernatants of B cells from DRB5 and DR3 nonallergic (3 subjects, 10 clones; Fig 4, C) and allergic subjects without cat ownership (3 subjects, 14 clones; Fig 4, A) or with cat ownership (3 subjects, 16 clones; Fig 4, B) in the presence or absence of Fel d 1 chain 1₁₇₋₃₆- or Fel d 1 chain 2₁₇₋₃₆-specific CD4⁺ T clones from DRB5 or DR3 allergic subjects without a cat with or without the Fel d 1 chain 1₁₇₋₃₆ or Fel d 1 chain 2₁₇₋₃₆ peptide. **D-F**, CD38 expression on B cells is shown in the *bottom panels*. Each clone was tested on duplicate or triplicate wells, and a minimum of 4 experiments were done per group. Data were compared by using the Mann-Whitney test. ****P* ≤ .001.

response manifested by an increase in specific IgG₄ levels is only observed in the chronic allergen exposure group. Therefore we speculated on a direct role for Fel d 1 protein in inducing IgG₄ production in subjects with cat allergy.

B cells from allergic subjects had already undergone IgG₄ class-switching and are potentially allergen experienced (Fig 4), and therefore we tested their capacity to produce IgG₄ on Fel d 1 recombinant protein activation. B cells isolated from allergic subjects without cat ownership were capable of producing IgG₄ in response to Fel d 1 only in the presence of sT_H2 clones (Fig 5, A). These results suggest that T_H2 cells support IgG₄ production through direct interaction with allergen-experienced B cells after they encounter the allergen.

On the other hand, B cells from nonallergic subjects were incapable of producing IgG₄ in the presence of sT_H2 clones and Fel d 1 protein (Fig 5, B). Only concomitant culture of B cells of nonallergic subjects with Fel d 1, sT_H2 clones, and antigen-presenting cells (ie, PBMCs depleted of B cells) was sufficient to induce IgG₄ (Fig 5, C). These experiments were Fel d 1 specific because IgG₄ was not induced in the presence of Fel d 4 (40 µg/mL, see Fig E12 in this article's Online Repository at www.jacionline.org). These experiments suggest that T_H2 cells cooperate with cells from the innate immune system to induce IgG₄ class-switching.

DISCUSSION

The cat allergen-specific T-cell response is characterized by a higher frequency of Fel d 1 and Fel d 4 sT_H2 cells in allergic subjects than in nonallergic subjects. High frequencies of Fel d 7-specific memory T_H2 CD4⁺ T-cell responses were only observed in approximately 10% of our cohort. A correlation between the frequency of Fel d 1 and Fel d 4 sT_H2 cells and IgE levels was observed in the group without a cat at home. A higher frequency of sT_H2 cells is also linked to an increase in asthma prevalence in subjects without cat ownership. These correlations were not observed in the group with cat ownership, and the frequency of Fel d 1 and Fel d 4 sT_H2 cells correlated only with specific IgG₄ levels and is linked to low sensitization. At the T-cell level, we did not observe a difference in cytokine profiles between these 2 groups. However, the possibility of molecular modifications of sT_H2 cells during chronic cat allergen exposure remains to be elucidated.

Earlier studies also demonstrated that both IL-10 and IL-4 regulate IgG₄ production.^{26,27} While IgG₄ is linked to decreased sensitivity, it is also known to correlate with cat allergen exposure in subjects with cat allergy.²⁸⁻³⁰ In accordance with this, we observed low allergen sensitivity in subjects with cat allergy and a cat at home. We also observed a direct correlation between sT_H2 cell frequency and specific IgG₄ levels for this group of

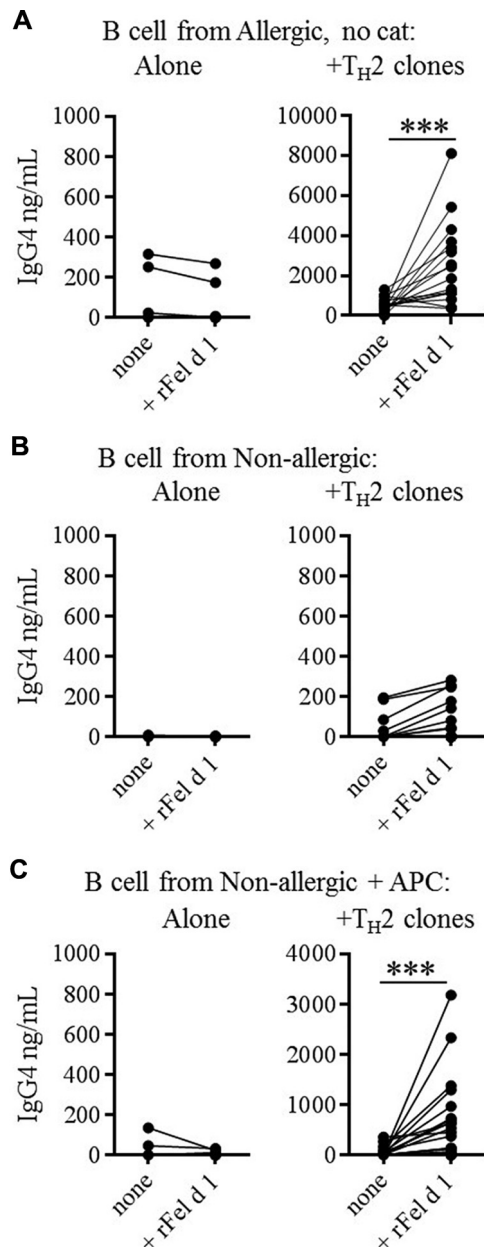


FIG 5. T_H2 cell-dependent IgG₄ production in response to Fel d 1 recombinant protein. Total IgG₄ levels in culture supernatants of B cells isolated from DRB5 and DR3 allergic (3 subjects, 11 clones; **A**) or nonallergic (3 subjects, 12 clones; **B** and **C**) subjects with or without CD4⁺ T_H2 clones and Fel d 1 recombinant protein (40 µg/mL) and with autologous PBMCs depleted of B cells (APC; Fig 5, **C**). Each clone was tested in duplicate or triplicate wells, and a minimum of 4 experiments were done per group. Data were compared by using the Mann-Whitney test. ****P* ≤ .05.

subjects. Moreover, we provide evidence that the cognate response (using peptide or whole protein) between Fel d 1 sT_H2 clones and B cells from allergic subjects is sufficient to induce IgG₄ production. This response might be mediated by allergen-experienced B cells after antigen recall, which have a capacity to rapidly differentiate into plasma cells.³¹ These results demonstrate the direct role of T_H2 cells in support of IgG₄ production and suggest that an IgG₄ response is part of a T_H2-driven response on allergen exposure. We speculate that the repeated stimulation of B cells by allergens and T_H2 cells

could explain the accumulation of IgG₄ during allergen exposure without major modification of the T_H2 response. We observed that IgG₄ accumulation on allergen exposure is related and dependent on the presence of T_H2 cells. However, T_H2 cells *in vivo*, in the absence of allergen exposure, do not seem to have the capacity to mediate IgG₄ accumulation, indicating a major role of repeated allergen exposure in this phenomenon.

On the other hand, T_H2 cells were not able to directly induce IgG₄ class-switching on B cells from nonallergic subjects (allergen-unexperienced B cells), implicating multiple signaling requirements for B cells to induce IgG₄ class-switching. Immunoglobulin class-switching can be induced by T cell-dependent and T cell-independent signals.³²⁻³⁵ For allergen-unexperienced B cells from nonallergic subjects, activation of the innate response by Fel d 1 is essential for T_H2 cell-dependent IgG₄ production. This phenomenon might underlie the primary induction of IgG₄ after initial encounter with allergen. Although the innate response alone is not sufficient to induce IgG₄ class-switching, innate cells still play an important role in T cell-dependent immunoglobulin class-switching.

The ability of some allergens to directly activate innate cells is already known.^{36,37} Hence, the phenomenon observed here likely applies to other allergens, such as Fel d 4. In fact it has been described for the protein Can f 6 (a lipocalin protein similar to Fel d 4), which can also activate innate cells.^{14,36,37}

The previously described major T cell-dependent immunoglobulin response is mediated by T_{FH} cells^{31,34,38}; however, in this study we were unable to detect CXCR5 expression by sT_H2 cells, suggesting T_{FH} cells are not involved in specific IgG₄ production on allergen exposure. Moreover, *ex vivo* assay of human sera reveals an acute accumulation of IgG₄ only during exposure.^{28,30} These observations suggest IgG₄ is not produced by long-lived plasma cells and is independent of the germinal center pathway. Transient production of IgG₄ can be explained by differentiation of IgG₄ B cells into short-lived extrafollicular plasma cells localized in the marginal zone.³⁹

This work provides new insights into the anti-inflammatory role of IgG₄ at the cellular level and into how chronic allergen exposure can modulate the immunoglobulin response. IgG₄ accumulation has previously been observed during chronic parasite infection, where it led to immunoregulation and a hyporesponse.⁴⁰⁻⁴² These observations, combined with the results described here, suggest that chronic activation of T_H2 cells and B cells (by allergen or parasites) mediates IgG₄ accumulation.

The phenomenon we describe here can also occur during allergen immunotherapy, during which an increase in specific IgG₄ levels could mediate the decrease in allergic symptoms without induction of T-cell tolerance. Indeed, IgG₄ accumulation during allergen immunotherapy is correlated with a decrease in allergic symptoms.^{10,11,43-45}

In summary, both asthma and cat allergen exposure are linked to a high frequency of T_H2 cells. We further demonstrate that during chronic cat allergen exposure, sT_H2 cells support IgG₄ production by allergen-experienced B cells, which in turn drives desensitization.

We thank Jennifer Heaton, Kavitha Gilroy, and Sylvia Posso for help with subject recruitment. We thank Eddie James and the Tetramer Core Laboratory at Benaroya Research Institute for production of tetramers. We thank Nadia Torres-Chinn for help with blood processing. Finally, we thank Michael Mason for help with statistical analysis.

Key messages

- Chronic cat allergen exposure is related to low sensitization.
- Correlation between sT_H2 cell accumulation and IgG₄ levels during chronic cat allergen exposure was observed.
- sT_H2 cells support IgG₄ production by allergen-experienced B cells in response to Fel d 1.

REFERENCES

- Pulendran B, Artis D. New paradigms in type 2 immunity. *Science* 2012;337:431-5.
- Platts-Mills T, Vaughan J, Squillace S, Woodfolk J, Sporik R. Sensitisation, asthma, and a modified Th2 response in children exposed to cat allergen: a population-based cross-sectional study. *Lancet* 2001;357:752-6.
- Erwin EA, Wickens K, Custis NJ, Siebers R, Woodfolk J, Barry D, et al. Cat and dust mite sensitivity and tolerance in relation to wheezing among children raised with high exposure to both allergens. *J Allergy Clin Immunol* 2005;115:74-9.
- Aalberse RC, Stapel SO, Schuurman J, Rispens T. Immunoglobulin G4: an odd antibody. *Clin Exp Allergy* 2009;39:469-77.
- van der Neut Kolfschoten M, Schuurman J, Losen M, Bleeker WK, Martinez-Martinez P, Vermeulen E, et al. Anti-inflammatory activity of human IgG4 antibodies by dynamic Fab arm exchange. *Science* 2007;317:1554-7.
- Uersimi C, Zabel F, Manolova V, Bauer M, Beerli RR, Senti G, et al. IgG-mediated down-regulation of IgE bound to mast cells: a potential novel mechanism of allergen-specific desensitization. *Allergy* 2014;69:338-47.
- Varga EM, Kausar F, Aberer W, Zach M, Eber E, Durham SR, et al. Tolerant beekeepers display venom-specific functional IgG4 antibodies in the absence of specific IgE. *J Allergy Clin Immunol* 2013;131:1419-21.
- Reefer AJ, Carneiro RM, Custis NJ, Platts-Mills TA, Sung SS, Hammer J, et al. A role for IL-10-mediated HLA-DR7-restricted T cell-dependent events in development of the modified Th2 response to cat allergen. *J Immunol* 2004;172:2763-72.
- Meiler F, Zumkehr J, Klunker S, Ruckert B, Akdis CA, Akdis M. In vivo switch to IL-10-secreting T regulatory cells in high dose allergen exposure. *J Exp Med* 2008;205:2887-98.
- Francis JN, James LK, Paraskevopoulos G, Wong C, Calderon MA, Durham SR, et al. Grass pollen immunotherapy: IL-10 induction and suppression of late responses precedes IgG4 inhibitory antibody activity. *J Allergy Clin Immunol* 2008;121:1120-5.e2.
- Mobs C, Ipsen H, Mayer L, Slotsch C, Petersen A, Wurtzen PA, et al. Birch pollen immunotherapy results in long-term loss of Bet v 1-specific TH2 responses, transient TR1 activation, and synthesis of IgE-blocking antibodies. *J Allergy Clin Immunol* 2012;130:1108-16.e6.
- Akdis CA, Akdis M. Mechanisms of immune tolerance to allergens: role of IL-10 and Tregs. *J Clin Invest* 2014;124:4678-80.
- Gronlund H, Saarne T, Gafvelin G, van Hage M. The major cat allergen, Fel d 1, in diagnosis and therapy. *Int Arch Allergy Immunol* 2010;151:265-74.
- Smith W, Butler AJ, Hazell LA, Chapman MD, Pomes A, Nickels DG, et al. Fel d 4, a cat lipocalin allergen. *Clin Exp Allergy* 2004;34:1732-8.
- Smith W, O'Neil SE, Hales BJ, Chai TL, Hazell LA, Tanyaratrisakul S, et al. Two newly identified cat allergens: the von Ebner gland protein Fel d 7 and the latherin-like protein Fel d 8. *Int Arch Allergy Immunol* 2011;156:159-70.
- Hales BJ, Chai LY, Hazell L, Elliot CE, Stone S, O'Neil SE, et al. IgE and IgG binding patterns and T-cell recognition of Fel d 1 and non-Fel d 1 cat allergens. *J Allergy Clin Immunol Pract* 2013;1:656-65, e1-5.
- Wambre E, DeLong JH, James EA, LaFond RE, Robinson D, Kwok WW. Differentiation stage determines pathologic and protective allergen-specific CD4+ T-cell outcomes during specific immunotherapy. *J Allergy Clin Immunol* 2012;129:544-51, e1-7.
- Kwok WW, Roti M, DeLong JH, Tan V, Wambre E, James EA, et al. Direct ex vivo analysis of allergen-specific CD4+ T cells. *J Allergy Clin Immunol* 2010;125:1407-9.e1.
- Renand A, Newbrough S, Wambre E, DeLong JH, Robinson D, Kwok WW. Arginine kinase Pen m 2 as an important shrimp allergen recognized by T2 cells. *J Allergy Clin Immunol* 2014;134:1456-9.e7.
- Chattopadhyay PK, Yu J, Roederer M. A live-cell assay to detect antigen-specific CD4+ T cells with diverse cytokine profiles. *Nat Med* 2005;11:1113-7.
- Bacher P, Schink C, Teutschbein J, Kniemeyer O, Assenmacher M, Brakhage AA, et al. Antigen-reactive T cell enrichment for direct, high-resolution analysis of the human naive and memory Th cell repertoire. *J Immunol* 2013;190:3967-76.
- Jones MG. Measurement of specific IgG anti-Fel d 1 antibodies. *Methods Mol Med* 2008;138:247-54.
- Wambre E, DeLong JH, James EA, Torres-Chinn N, Pfutzner W, Mobs C, et al. Specific immunotherapy modifies allergen-specific CD4(+) T-cell responses in an epitope-dependent manner. *J Allergy Clin Immunol* 2014;133:872-9.e7.
- Morita R, Schmitt N, Benteibibel SE, Ranganathan R, Bourdery L, Zurawski G, et al. Human blood CXCR5(+)CD4(+) T cells are counterparts of T follicular cells and contain specific subsets that differentially support antibody secretion. *Immunity* 2011;34:108-21.
- Benteibibel SE, Lopez S, Obermoser G, Schmitt N, Mueller C, Harrod C, et al. Induction of ICOS+CXCR3+CXCR5+ TH cells correlates with antibody responses to influenza vaccination. *Sci Transl Med* 2013;5:176ra32.
- Jeannin P, Lecoanet S, Delneste Y, Gauchat JF, Bonnefoy JY. IgE versus IgG4 production can be differentially regulated by IL-10. *J Immunol* 1998;160:3555-61.
- Gascan H, Gauchat JF, Roncarolo MG, Yssel H, Spits H, de Vries JE. Human B cell clones can be induced to proliferate and to switch to IgE and IgG4 synthesis by interleukin 4 and a signal provided by activated CD4+ T cell clones. *J Exp Med* 1991;173:747-50.
- Erwin EA, Woodfolk JA, James HR, Satinover SM, Platts-Mills TA. Changes in cat specific IgE and IgG antibodies with decreased cat exposure. *Ann Allergy Asthma Immunol* 2014;112:545-50.e1.
- Burnett M, Wegienka G, Havstad S, Kim H, Johnson CC, Ownby D, et al. Relationship of dog- and cat-specific IgE and IgG4 levels to allergic symptoms on pet exposure. *J Allergy Clin Immunol Pract* 2013;1:350-3.
- Oryszczyn MP, Van Ree R, Maccario J, Nadif R, Kauffmann F. EGEA cooperative group. Cat sensitization according to cat window of exposure in adult asthmatics. *Clin Exp Allergy* 2009;39:1515-21.
- McHeyzer-Williams M, Okitsu S, Wang N, McHeyzer-Williams L. Molecular programming of B cell memory. *Nat Rev Immunol* 2012;12:24-34.
- Xu Z, Zan H, Pone EJ, Mai T, Casali P. Immunoglobulin class-switch DNA recombination: induction, targeting and beyond. *Nat Rev Immunol* 2012;12:517-31.
- Cerutti A, Zan H, Schaffer A, Bergsagel L, Harindranath N, Max EE, et al. CD40 ligand and appropriate cytokines induce switching to IgG, IgA, and IgE and coordinated germinal center and plasmacytoid phenotypic differentiation in a human monoclonal IgM+IgD+ B cell line. *J Immunol* 1998;160:2145-57.
- Goodnow CC, Vinuesa CG, Randall KL, Mackay F, Brink R. Control systems and decision making for antibody production. *Nat Immunol* 2010;11:681-8.
- Pone EJ, Xu Z, White CA, Zan H, Casali P. B cell TLRs and induction of immunoglobulin class-switch DNA recombination. *Front Biosci* 2012;17:2594-615.
- Herre J, Gronlund H, Brooks H, Hopkins L, Waggoner L, Murton B, et al. Allergens as immunomodulatory proteins: the cat dander protein Fel d 1 enhances TLR activation by lipid ligands. *J Immunol* 2013;191:1529-35.
- Bublin M, Eiwegger T, Breiteneder H. Do lipids influence the allergic sensitization process? *J Allergy Clin Immunol* 2014;134:521-9.
- Vinuesa CG, Chang PP. Innate B cell helpers reveal novel types of antibody responses. *Nat Immunol* 2013;14:119-26.
- Cerutti A, Cols M, Puga I. Marginal zone B cells: virtues of innate-like antibody-producing lymphocytes. *Nat Rev Immunol* 2013;13:118-32.
- Adjomey T, Hoerauf A. Induction of immunoglobulin G4 in human filariasis: an indicator of immunoregulation. *Ann Trop Med Parasitol* 2010;104:455-64.
- Yazdanbakhsh M, Kremsner PG, van Ree R. Allergy, parasites, and the hygiene hypothesis. *Science* 2002;296:490-4.
- Figueiredo CA, Barreto ML, Rodrigues LC, Cooper PJ, Silva NB, Amorim LD, et al. Chronic intestinal helminth infections are associated with immune hypo-responsiveness and induction of a regulatory network. *Infect Immun* 2010;78:3160-7.
- James LK, Shamji MH, Walker SM, Wilson DR, Wachholz PA, Francis JN, et al. Long-term tolerance after allergen immunotherapy is accompanied by selective persistence of blocking antibodies. *J Allergy Clin Immunol* 2011;127:509-16, e1-5.
- James LK, Bowen H, Calvert RA, Dodev TS, Shamji MH, Beavil AJ, et al. Allergen specificity of IgG(4)-expressing B cells in patients with grass pollen allergy undergoing immunotherapy. *J Allergy Clin Immunol* 2012;130:663-70.e3.
- Akdis M, Akdis CA. Mechanisms of allergen-specific immunotherapy: multiple suppressor factors at work in immune tolerance to allergens. *J Allergy Clin Immunol* 2014;133:621-31.

METHODS

HLA typing

All subjects were HLA typed by using sequence-specific oligonucleotide primers with UniTray SSP Kits (Invitrogen, Carlsbad, Calif).

Asthma and rhinitis scores

For rhinitis, the score is based on the use of medication and cat avoidance: mild rhinitis is defined as a positive skin test response with or without antihistamine medication (10 mg of cetirizine, 10 mg of loratadine, or 180 mg of fexofenadine per day); moderate rhinitis was defined as nasal steroids (50 µg of fluticasone, 1 spray in each nostril twice per day or equivalent) and antihistamine (10 mg of cetirizine, 10 mg of loratadine, or 180 mg of fexofenadine per day); and severe rhinitis was defined as the same medication use as the moderate group but also the occasional requirement for higher dosages and the inability to tolerate cat exposure. For asthma, diagnosis is based on measurement of airflow obstruction by using spirometry, which is reversible with medications. Patients were classified based on the Global Initiative for Asthma classification and their medications. All subjects recruited had either intermittent, mild persistent, or moderate persistent symptoms, with short-acting β-agonist medication and a low to moderate dose of inhaled corticosteroids with or without a long-acting β-agonist.

Specific T-cell frequency formula

Frequency was determined by using the following formula:

$$F = n/N,$$

where n designates the number of CD154⁺ cells in the bound fraction after enrichment, and N is the total number of CD4⁺ T cells calculated as 10× the number of CD4⁺ T cells in a one-tenth fraction that was saved for analysis.

Fluorescent antibodies and flow cytometry

For phenotype and surface staining, PerCP-Cy5.5 anti-CD14 (HCD14; BioLegend, San Diego, Calif), PerCP-Cy5.5 anti-CD19 (HIB19, BioLegend), V500 anti-CD4 (RPA-T4, BD Biosciences), Alexa Fluor 700 anti-CD45RA

(HI100, BD Biosciences), Alexa Fluor 488 anti-CD183 (CXCR3, G025H7, BioLegend), PE-Cy7 anti-CD194 (TG6/CCR4, BioLegend), Alexa Fluor 647 anti-CD294 (CRTH2, BM16, BD Biosciences), allophycocyanin (APC)–Cy7 anti-CD27 (O323, BioLegend), and Alexa Fluor 488 anti-CD185 (CXCR5, RF8B2, BD Biosciences) antibodies and Cell Viability Solution (BD Via-Probe, BD Biosciences) were used.

For intracellular staining, APC-Cy7 anti-CD4 (OKT3, BioLegend), Alexa Fluor 488 anti-IL-4 (8D4-8, eBioscience), Alexa Fluor 700 anti-IFN-γ (4S.B3, BioLegend), Alexa Fluor 647 anti-IL-21 (3A3-N2.1, BD Biosciences), APC anti-IL-5 (TRFK5, BD Biosciences), PerCP-Cy5.5 anti-IL-17 (BL168, BioLegend), and PE-Cy7 anti-IL-10 (JES3-9D7, BioLegend) antibodies and Fixable Viability Stain 450 (BD Biosciences) were used.

For surface staining during B-cell culture, Alexa Fluor 648 anti-CD38 (HB-7, BioLegend), Alexa Fluor 488 anti-CD19 (HIB19, BioLegend), and Brilliant Violet 421 (BV421) anti-CD4 (RPA-T4, BD Biosciences) and Cell Viability Solution (BD Via-Probe, BD Biosciences) were used.

Data were acquired on an LSR II flow cytometer (BD Biosciences) by using FACSDiva software (BD Biosciences) and analyzed with FlowJo 8.8.2 software (TreeStar, Ashland, Ore).

ELISA detection methods

For specific IgG₄ detection, we stained with Biotin Mouse Anti-Human IgG₄ (G17-4, BD Biosciences), followed by Europium-labeled streptavidin (PerkinElmer, Waltham, Mass), and plates were read on a Victor2 D time-resolved fluorometer (PerkinElmer). Europium fluorescence was measured by using time-resolved fluorometry.

Fel d 1 sT_H2 clone generation

Single cells were expended in the presence of 10⁵ autologous irradiated PBMCs, 1 µg/mL PHA (Sigma, St Louis, Mo) and human IL-2 (10 U/mL; Roche, Mannheim, Germany) for 5 days in 200 µL of culture medium on a 96-well plate. After 5 days, IL-2 and fresh medium were added each day. After 14 days, wells with clonally expanded cells were stimulated with 10⁶ irradiated PBMCs, 1 µg/mL PHA (Sigma), and human IL-2 (10 U/mL, Roche) for 5 days in a 48-well plate. An average of 96 cells were sorted *ex vivo*, and a maximum of 25 clones were obtained at the end. Ninety percent of these clones bind the tetramer.

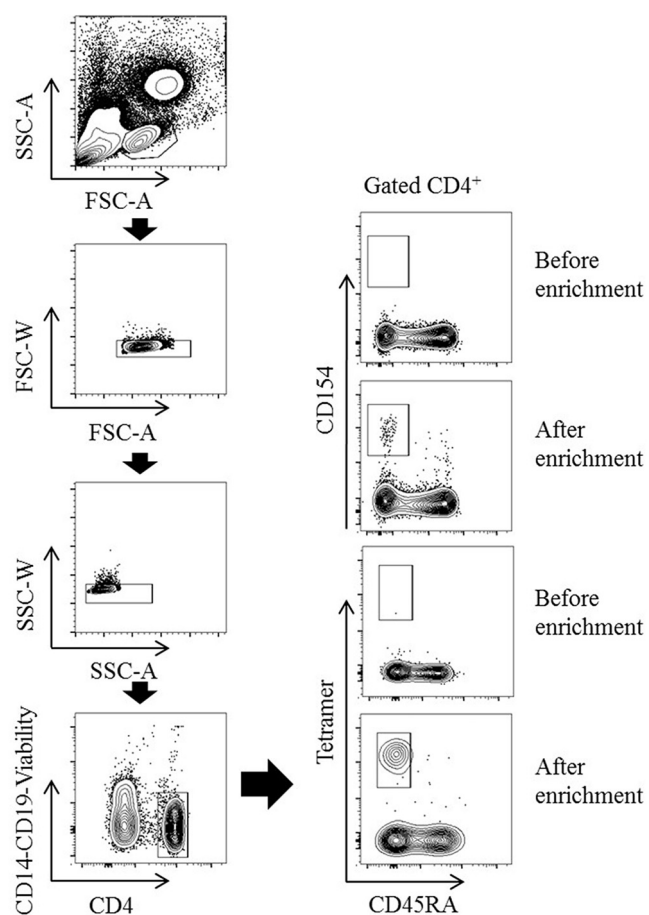


FIG E1. Gating strategies and methods for detection of allergen-specific CD4⁺ T cells. CD4⁺ T cells were gated after removing doublets and CD14⁺, CD19⁺, and dead cells (viability positive). PE-CD154⁺ or PE-peptide-MHC class II tetramer-positive cells were detected after anti-PE bead enrichment. FSC, Forward scatter; SSC, side scatter.

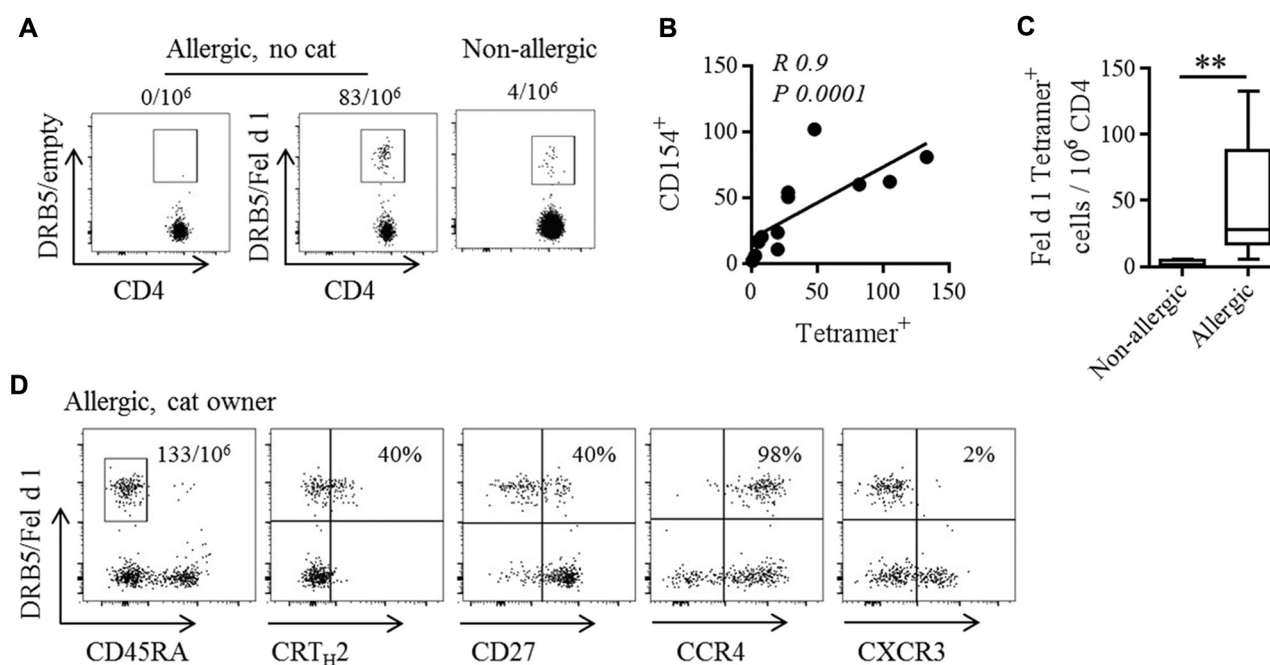


FIG E2. Characterization of the Fel d 1-specific T-cell response by using tetramer staining. **A**, DRB5/Fel d 1 chain 117-36 tetramer-positive CD4⁺ T cells in DRB5 nonallergic and allergic subjects. Frequencies of Fel d 1-specific CD4⁺ T cells per million CD4⁺ T cells are as indicated. **B**, Correlation between the frequency of Fel d 1-specific memory CD154⁺CD45RA⁻CD4⁺ T cells and the frequency of DRB5/Fel d 1 chain 117-36 and DRB3/Fel d 1 chain 217-36 tetramer-positive CD4⁺ T cells in DRB5 and DR0301 allergic adults. **C**, Frequency of Fel d 1-specific CD4⁺ T cells in DRB5 and DR0301 nonallergic and allergic adults. **D**, DRB5/Fel d 1 chain 117-36 tetramer-positive CD4⁺ T cells in a DRB5 cat-owning allergic subject. Frequencies of Fel d 1-specific CD4⁺ T cells per million CD4⁺ T cells and percentages of CRTH2, CD27, CCR4, and CXCR3 on Fel d 1-specific CD45RA⁻CD4⁺ T cells are as indicated. Spearman R and P values were determined to analyze the correlation. Data were compared by using the Mann-Whitney test. ** $P \leq .01$.

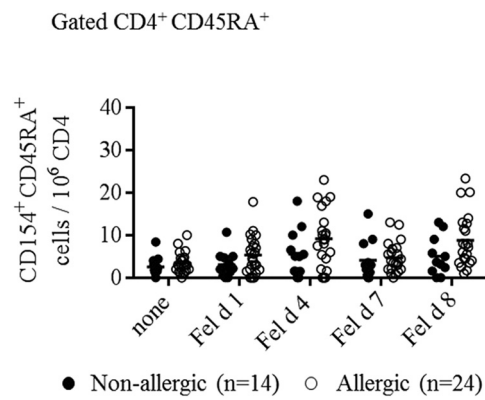


FIG E3. Frequency of cat allergen-specific naive CD45RA⁺CD4⁺ T cells in allergic and nonallergic subjects. Frequency of total naive CD45RA⁺CD154⁺CD4⁺ T cells after cat allergen peptide stimulation of PBMCs in 14 nonallergic and 24 allergic subjects.

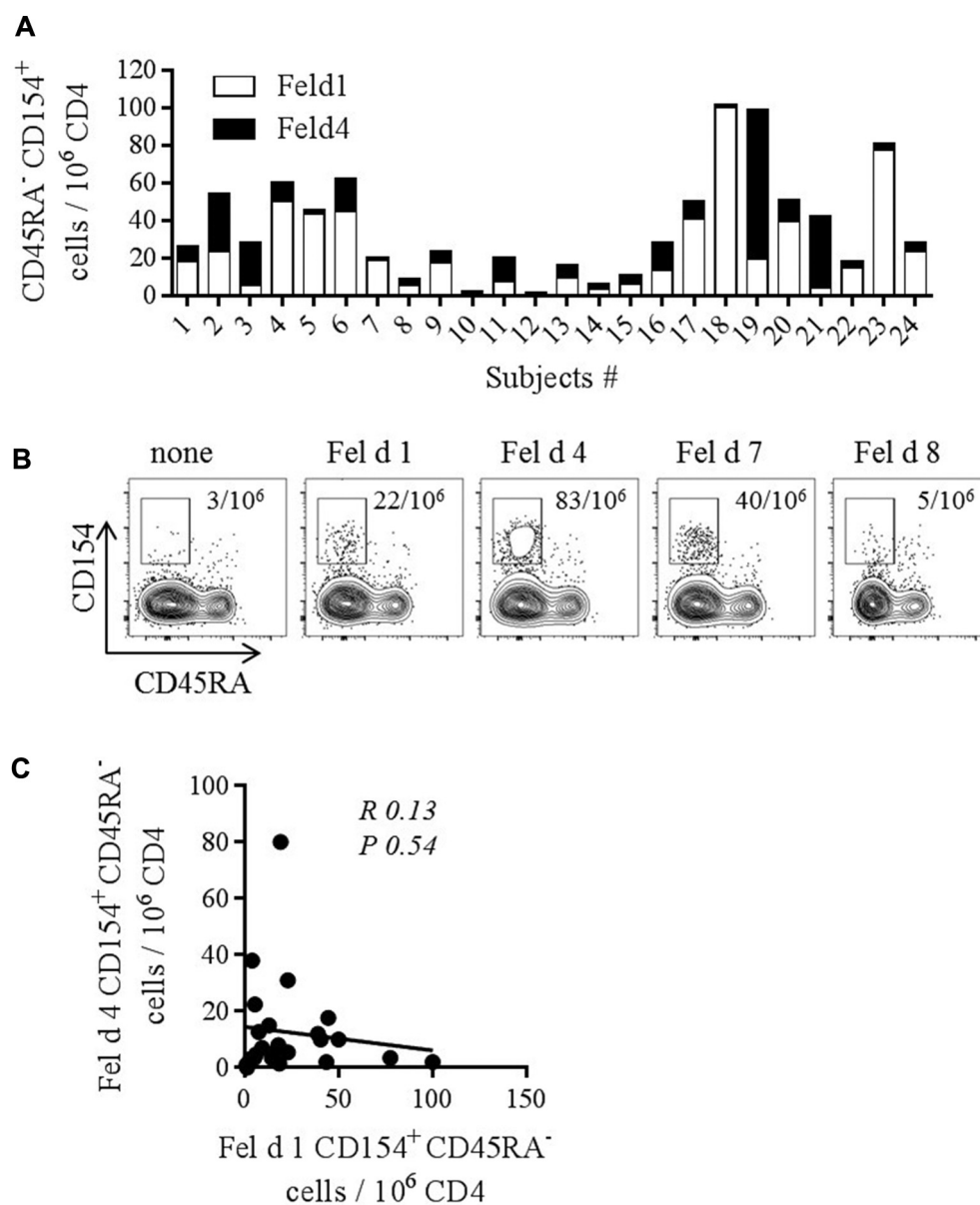
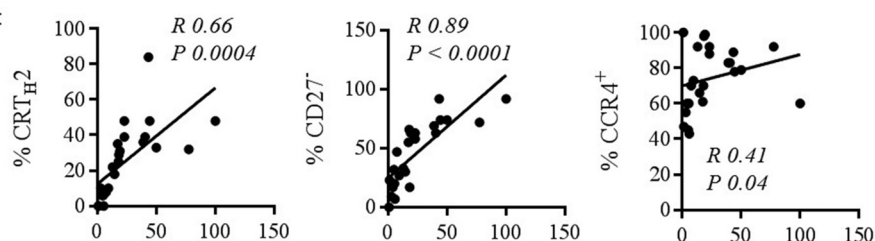


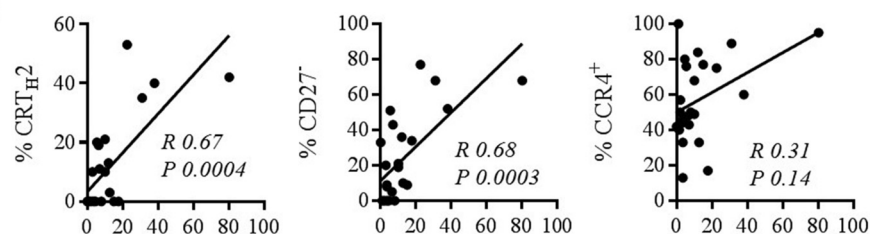
FIG E4. Frequencies of Fel d 1 and Fel d 4 sT_H2 cells do not correlate during cat allergy. **A**, Frequencies of Fel d 1- and Fel d 4-specific memory CD154⁺ T cells in each allergic subject. **B**, CD154 expression on gated CD4⁺ T cells after 3 hours of PBMC stimulation with or without cat allergen peptides. Results shown are after anti-PE bead enrichment in an allergic subject with a strong response against Fel d 4. Numbers indicate frequencies of CD154⁺CD4⁺ T cells per 10⁶ CD4⁺ T cells. **C**, Absence of correlation between frequencies of Fel d 1- and Fel d 4-specific memory CD154⁺CD45RA⁻CD4⁺ T cells in allergic subjects. Spearman *R* and *P* values were determined to analyze the correlation.

A

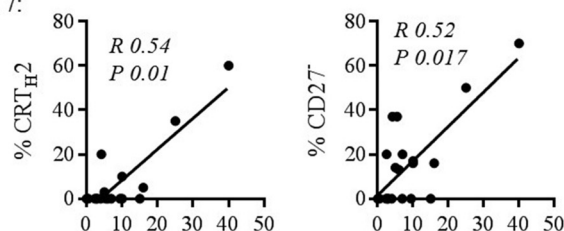
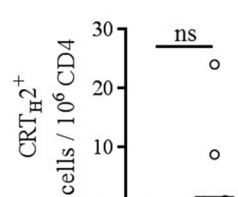
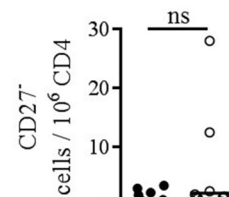
Fel d 1:



Fel d 4:

CD45RA⁻ CD154⁺ cells / 10⁶ CD4**B**

Fel d 7:

CD45RA⁻ CD154⁺ cells / 10⁶ CD4**C****D**

● Non-allergic ○ Allergic

FIG E5. T_H2 phenotype of Fel d 1-, Fel d 4-, and Fel d 7-specific memory T cells during cat allergy. **A**, Correlation between the frequency of memory CD154⁺CD45RA⁻CD4⁺ T cells and the percentage of CRTH2⁺, CD27⁻, and CCR4⁺ cells in CD154⁺CD45RA⁻ memory CD4⁺ T cells after Fel d 1 or Fel d 4 peptide stimulation of allergic PBMCs. **B**, Correlation between the frequency of memory CD154⁺CD45RA⁻CD4⁺ T cells and the percentage of CRTH2⁺ and CD27⁻ cells in CD154⁺CD45RA⁻ memory CD4⁺ T cells after Fel d 7 peptide stimulation of allergic PBMCs. **C** and **D**, Frequency comparison of Fel d 7-specific memory CD154⁺CRTH2⁺ (Fig E5, C) or CD27⁻ (Fig E5, D) CD4⁺ T cells between 24 allergic and 14 nonallergic subjects. Spearman *R* and *P* values were determined to analyze the correlation. Data were compared by using the Mann-Whitney test. *ns*, Not significant.

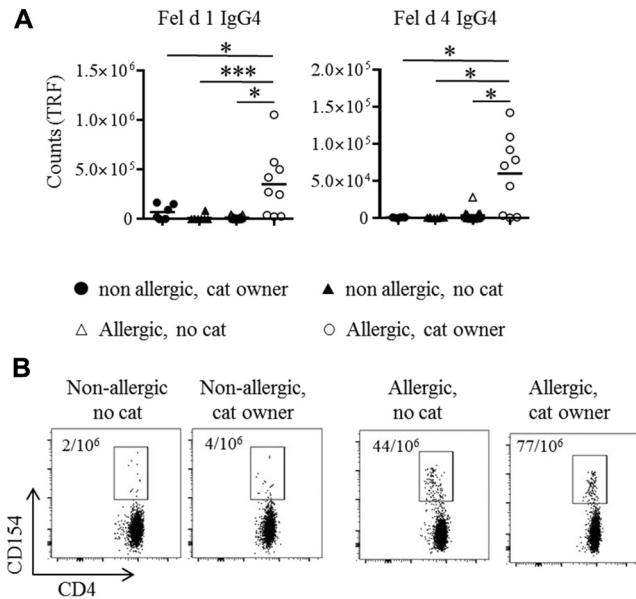


FIG E6. Fel d 1- and Fel d 4-specific IgG₄ and Fel d 1-specific T-cell responses in relation to allergic status and cat ownership. **A**, Fel d 1-specific (left) and Fel d 4-specific (right) IgG₄ levels from the serum of nonallergic subjects with or without a cat and allergic adults with or without a cat. TRF, Time-resolved fluorometry. **B**, Frequency of Fel d 1-specific memory CD154⁺CD45RA⁻, CD4⁺ T cells in nonallergic subjects without or with a cat, non-cat-owning allergic adults, and cat-owning allergic adults. Numbers indicate the frequency of CD154⁺CD4⁺ T cells per 10⁶ CD4⁺ T cells. Data were compared by using the Kruskal-Wallis test with the Dunn multiple comparisons test. * $P \leq .05$ and *** $P \leq .001$.

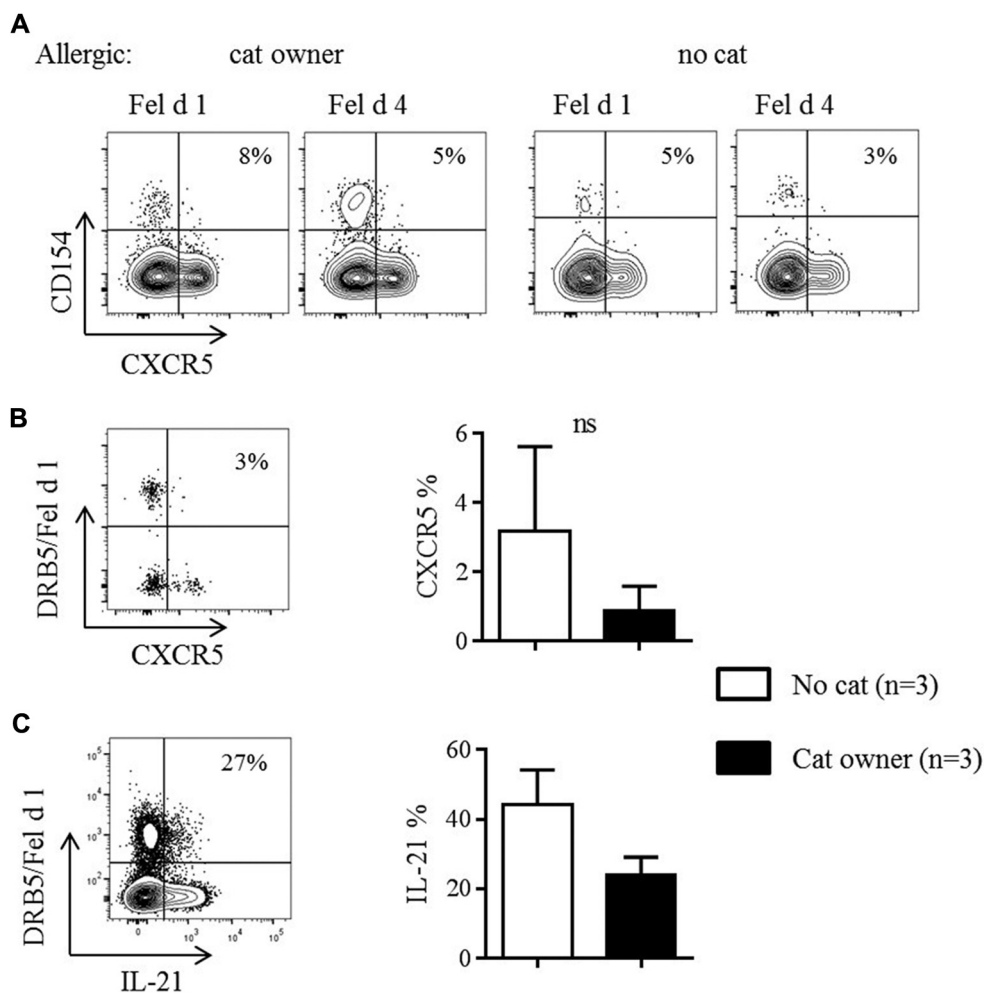


FIG E7. Analysis of T_{FH} cell markers (CXCR5 and IL-21) on cat allergen-specific $CD4^+$ T cells. **A**, CXCR5 and CD154 expression on gated memory $CD45RA^-CD4^+$ T cells from a cat-owning (*left*) or non-cat-owning (*right*) allergic adult. The percentage of CXCR5 $^+$ cells on $CD154^+CD45RA^-CD4^+$ T cells is as indicated. **B**, Fel d 1 chain 1 $_{17-36}$ -specific $CD4^+$ T cells in a DRB5 cat-owning allergic subject. Percentage of CXCR5 on Fel d 1-specific $CD45RA^-CD4^+$ T cells is as indicated. **C**, Intracellular IL-21 staining of Fel d 1 chain 1 $_{17-36}$ -specific $CD4^+$ T cells in a DRB5 cat-owning allergic adult. Percentages of IL-21 $^+$ Fel d 1-specific $CD4^+$ T cells are indicated. Data were compared by using the Mann-Whitney test. *ns*, Nonsignificant.

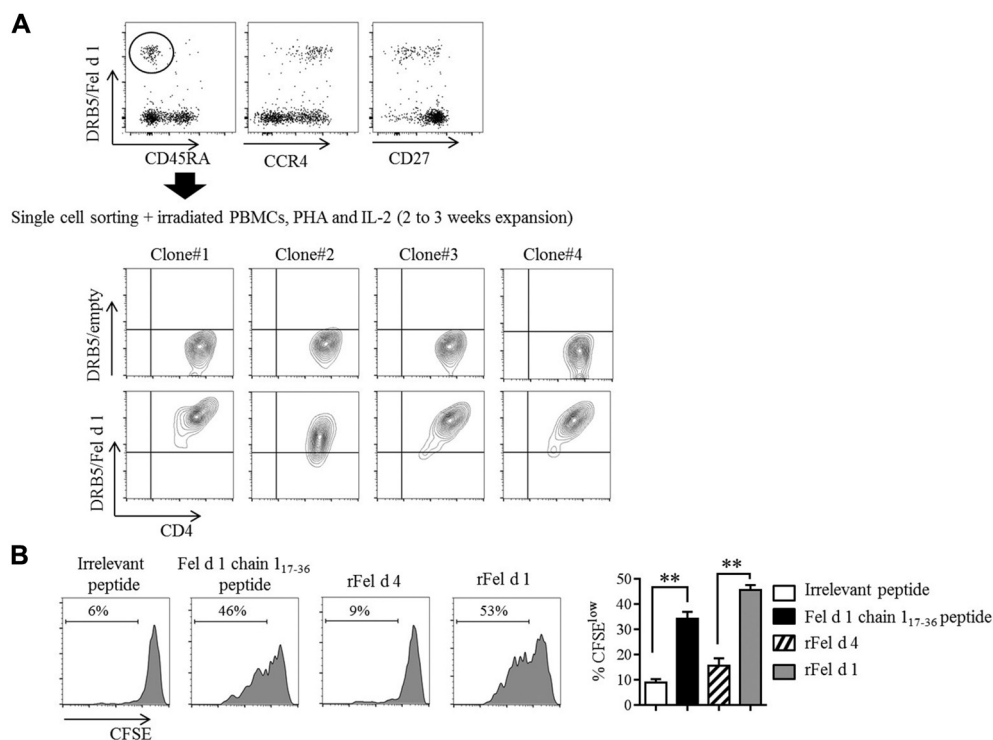


FIG E8. Selection of Fel d 1 chain 1₁₇₋₃₆-specific CD4⁺ T clones after single-cell sorting and expansion. **A**, Fel d 1 chain 1₁₇₋₃₆-specific CD4⁺ T clones were selected based on their capacity to bind DRB5/Fel d 1 tetramer compared by using DRB5/empty tetramer. Dot plots represent examples of positive staining of 4 different Fel d 1 chain 1₁₇₋₃₆-specific CD4⁺ T clones by using the DRB5/empty (*top*) and DRB5/Fel d 1 (*bottom*) tetramer. **B**, Specificity of clones ($n = 3$) was also confirmed by using a proliferation assay (carboxyfluorescein succinimidyl ester [CFSE] dilution) with irrelevant peptide (Fel d 1 chain 1₁₋₂₀ peptide), Fel d 1 chain 1₁₇₋₃₆ peptide, Fel d 4 recombinant protein (rFel d 4), or Fel d 1 recombinant protein (rFel d 1) after 4 days of culture. Similar data were obtained with Fel d 1 chain 2₁₇₋₃₆ CD4⁺ T-cell clones derived from DR3 allergic subjects. ** $P \leq .01$.

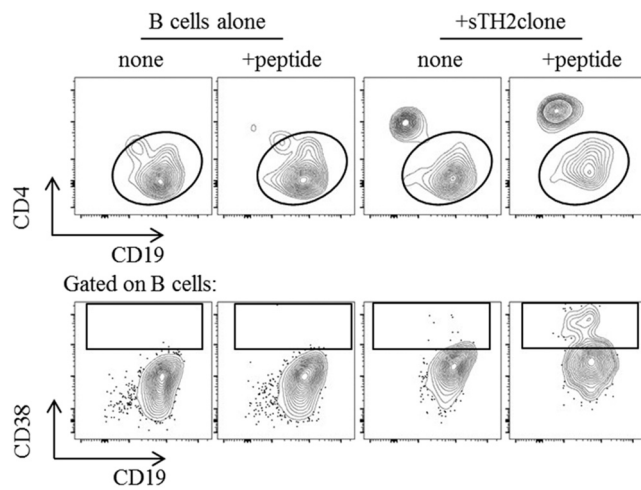


FIG E9. Gating strategy for CD38 expression on B cells during coculture with T_H2 clones and peptide. CD38 expression on gated CD19⁺ B cells during culture with or without T_H2 clones and peptides.

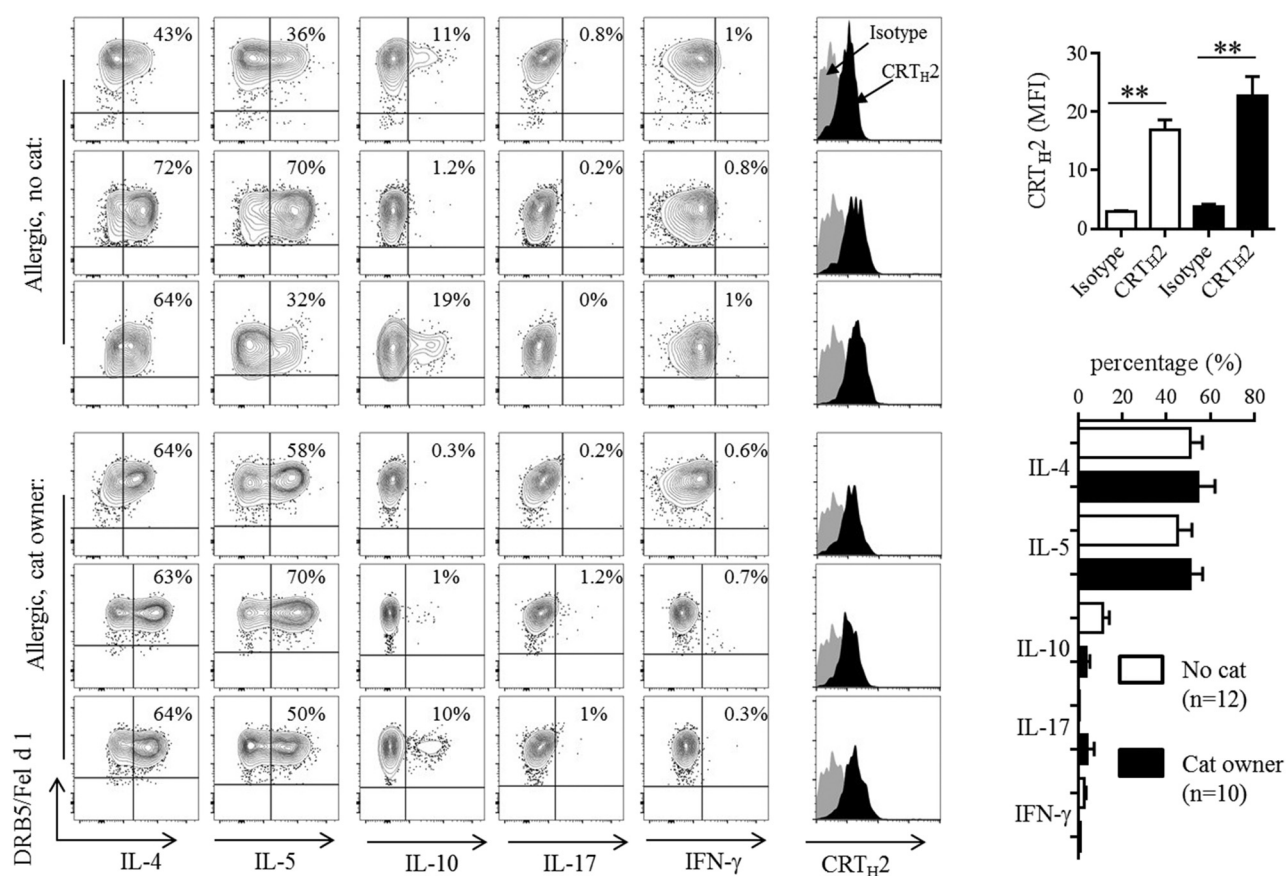


FIG E10. T_H2 profile of Fel d 1 chain 1₁₇₋₃₆-specific CD4⁺ T-cells clones from allergic adults with or without a cat. Fel d 1 chain 1₁₇₋₃₆-specific CD45RA⁺ CD4⁺ T cells from DRB5 allergic subjects with or without a cat were *ex vivo* sorted and expanded *in vitro* (single-cell sorting). Dot plots represent examples of intracellular cytokine staining of 6 different Fel d 1 chain 1₁₇₋₃₆-specific CD4⁺ T-cell clones. Percentages of IL-4, IL-5, IL-10, IFN- γ , IL-17, and CD4 Fel d 1-specific T-cell clones are indicated. Histograms show CRTH2 expression (black) compared with isotype antibody (gray). Graphs summarize the CRTH2 mean fluorescence intensity (MFI) and cytokine profiles of T_H2 clones from allergic adults with (10 clones) or without (12 clones) cat ownership. Data were compared by using the Mann-Whitney test. ** $P \leq .01$.

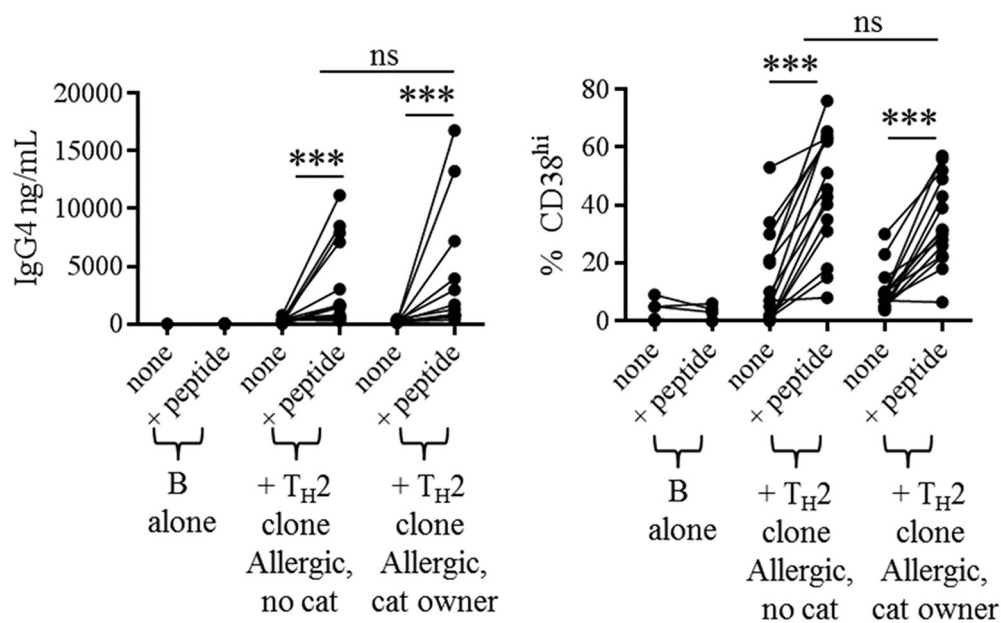


FIG E11. Fel d 1 sT_H2 clones from allergic adults with or without cat ownership support IgG₄ production by B cells from allergic adults. Total IgG₄ levels in the culture supernatant (*left*) and CD38 expression (*right*) on B cells from DRB5 or DR3 allergic subjects in the presence or absence of Fel d 1 chain 1₁₇₋₃₆ or Fel d 1 chain 2₁₇₋₃₆-specific CD4⁺ T-cell clones from DRB5 or DR3 allergic subjects without or with a cat with or without the Fel d 1 peptide (3 subjects, 7 clones). Each clone was tested on duplicate or triplicate wells. Data were compared by using the Mann-Whitney test. *** $P \leq .001$. ns, Not significant.

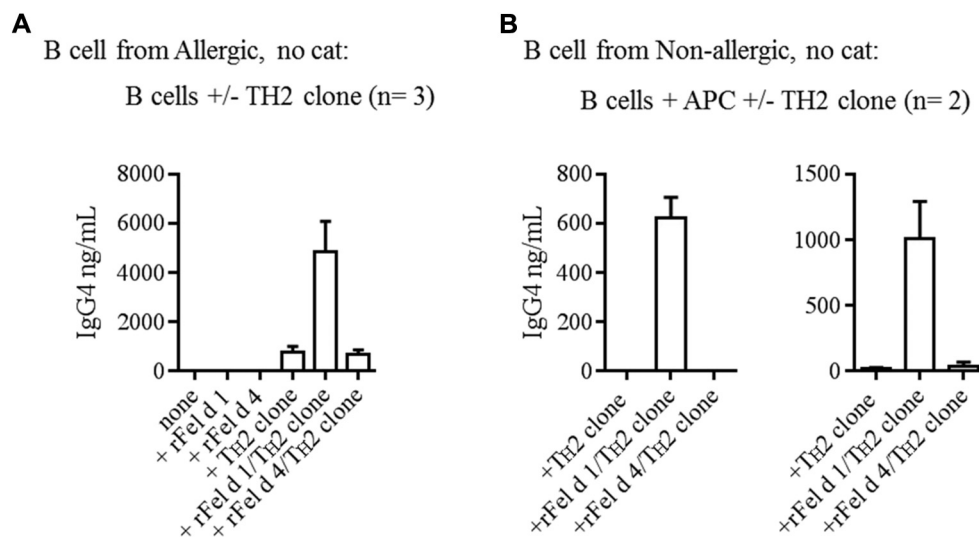


FIG E12. Antigen specificity of Fel d 1 sT_H2 cells in induction of IgG₄. Total IgG₄ levels were analyzed after 14 days in isolated B-cell culture from DRB5 allergic (**A**) or nonallergic subjects (without a cat; **B**) with or without autologous PBMCs (APC) depleted of B cells with or without DRB5 Fel d 1 chain 1₁₇₋₃₆-specific CD4⁺ T_H2 clones, Fel d 1 (40 µg/mL), or Fel d 4 (40 µg/mL).